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LIPID COMPONENTS AND *IN VITRO* MINERALIZATION OF SOME INVERTEBRATE CARTILAGES

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Light and electron microscopic studies (Schaffer, 1930; Godman and Porter, 1960) as well as chemical investigations (see reviews by Irving and Wuthier, 1968, and Wuthier, 1973) have established the existence of significant lipid components in cartilages of vertebrate origin. Preliminary light and electron microscopic and chemical data have also indicated the presence of significant lipid components in invertebrate cartilage tissues (Person and Philpott, 1969a, b). Insofar as we are aware, however, there are no detailed analytical chemical data in the literature for invertebrate cartilage tissue lipid components. The present investigation was therefore undertaken as a beginning effort to provide such data.

In the course of the above investigation, as will be seen, it was found that the odontophore cartilage of *Busycon canaliculatum* was characterized by a high content of phosphatidyl serine. This observation was of great interest because it is now known that phosphatidyl serine plays an important role in both *in vivo* and *in vitro* mineralization of vertebrate cartilages (Irving and Wuthier, 1968; Wuthier, 1973). While invertebrate cartilages do not mineralize *in vivo* (Schaffer, 1930; Person and Philpott, 1969a), we have recently established by chemical and X-ray diffraction studies that *Limulus* gill cartilage can be induced to mineralize *in vitro* by incubation in a solution metastable for hydroxyapatite (Eilberg, Person and Zuckerberg, 1975). We have also found in unpublished experiments that other invertebrate cartilages will mineralize *in vitro* in the same hydroxyapatite-metastable medium (*i.e., Loligo* cranial or head cartilage and *Busycon* odontophore cartilage). The present study was therefore extended to include preliminary observations designed to detect possible relationships between the *in vitro* mineralizability of invertebrate cartilages and their phosphatidyl serine content.

Materials and Methods

Tissue preparation for lipid analyses

Living specimens of the horse-shoe crab *Limulus polyphemus*, the marine snail, *Busycon canaliculatum*, and the squid *Loligo pealii* were obtained from the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts, in July, 1972. Also at this time, specimens of the "feather-duster" worm *Eudistylia*

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polymorpha were obtained from the Pacific Biomarine Supply Company, Venice, California, 90921. Animals were maintained in good physiological condition in running sea water tanks for 2–3 days prior to sacrifice. The following cartilage tissues were dissected and carefully trimmed of adherent tissues: gill cartilage from *Limulus* (8–10 inches body length); odontophore cartilage from *Busycon* (4–5 inches in shell length); cranial cartilage from *Loligo* (5–6 inches in mantle length); basal and tentacle cartilage of the crown of *Eudistylia* (4–6 inches in body length). Immediately following dissection and trimming, tissues were blotted with filter paper and wet weights were obtained. The tissues were then quickly cut into small pieces (3–4 mm on an edge) with scissors and sealed under nitrogen at atmospheric pressure, in glass vials containing a 2:1 (v/v) cold mixture of chloroform-methanol.

Analytical procedures

Fuller details and references for the analytical procedures outlined below are given in Rabinowitz, Luddy, Barford, Herb, Orlean, and Cohen (1967) and Rabinowitz, Bailey and Marsh (1971).

Lipids. All lipids or solutions containing lipids were kept under nitrogen at all times. Also, when stored, lipids were kept under nitrogen at −20° C in the presence of approximately 5 μg of butylated hydroxytoluene (BHT) per mg of lipid. Lipid standards were purchased from Applied Sciences, State College, Pennsylvania, and were tested for purity by duplicate chromatography, as described below. In no case did the level of impurities exceed 1%, and, in most instances, no detectable impurities were present. The tissue specimens were chopped finely and homogenized, following which they were extracted with chloroform-methanol (2:1, v/v). Only one extraction was required. Repeated extractions did not yield additional lipid material. One aliquot of the extract was subjected to silicic acid (Bio-Sil A, 100–200 mesh) column chromatography to separate neutral lipids (by chloroform elution) from phospholipids (by methanol elution). Individual lipid families were obtained from the separated neutral lipids and phospholipids by chromatography on thin layer plates and each was individually identified and assayed. Silica gel-G plates with petroleum ether-ethyl ether-acetic acid (90:10:1, v/v/v) were used for the nonpolar lipids. At both sides of the spot with the unknown, known amounts of various authentic standards were placed. After developing, the area corresponding to each standard was scraped from the plate. Silica gel-H with chloroform-methanol-acetic acid-water (200:120–25:15, v/v/v/v) was used for the polar lipids. Each of the neutral and phospholipid families were hydrolyzed with alkali, and the isolated fatty acids converted to methyl esters, which were assayed by gas-liquid chromatography. The column used was 10% diethylene glycol succinate polyester, in a $\frac{1}{8}$ in., 6 ft. column, maintained at 20 lb. pressure of argon. Temperature programming was at 1° C per minute starting at 140° C until 190° C was reached.

DNA, RNA and protein. After extraction of lipids, the residue was isolated by centrifugation and washed with 5 ml of cold 5% trichloroacetic acid (TCA). Five ml of 5% TCA were added to the remaining residue and the mixture heated at 90° C for 15 minutes. After cooling, the solution was filtered. The soluble material was transferred to a volumetric flask for use as the stock solution for the assays of collagen, DNA and RNA.

Non-collagen protein. The biuret technique was utilized for protein assays. These were done on 1 ml aliquots of the stock solution. Bovine serum albumin (Nutritional Biochemicals, Cleveland, Ohio) was used as the standard.

Collagen. Collagen was assayed by a micro-biuret method. "Tendon-collagen" (Sigma Chemical Company, St. Louis, Missouri) was used as the standard. Samples were hydrolyzed and hydroxyproline assayed by chromatography.

DNA and RNA. DNA was assayed by the diphenylamine reaction while RNA was determined using the orcinol technique. "Purified DNA and RNA" (Nutritional Biochemicals) were used as standards.

Mineralization experiments

Gill cartilages from *Limulus*, cranial cartilages from *Loligo* and odontophore cartilages from *Busycon* were dissected from live animals and trimmed of adherent extraneous tissues. Slices were cut by hand in dimensions of 0.5–3.0 mm thickness, and placed in a solution metastable for hydroxyapatite (Eilberg, Gould and Sobel, 1965), prepared as follows: to a one liter volumetric flask was added 100 ml of a solution containing $NaHCO_3$ (220 mM/l), KCl (50 mM/l) and NaCl (702 mM/l). Next, 50 ml of a solution containing Na_2HPO_4 (25.1 mM/l) and NaH_2PO_4 (6.45 mM/l as $NaH_2PO_4 \cdot H_2O$) was added. Following this, about 200 ml of distilled water was added. CO_2 was then bubbled through the solution for one minute. Next, 11 ml of a $CaCl_2$ solution (250 mM/l as $CaCl_2 \cdot 2H_2O$) was added, and the flask made to mark with distilled water. N_2 was bubbled through the resulting solution until a pH of 7.30 was reached. (Attempts to induce mineralization of invertebrate cartilages in calcium carbonate-metastable media have been unsuccessful thus far). Tissue incubations were carried out at 37° C for 10, 16, 24, 32, 48 and 96 hours, using 50 ml Erlenmeyer flasks, filled to the brim with the above medium, and closed with stopcock grease-sealed rubber stoppers. Control incubations were carried out in the same media at 20° C, at which temperature it has been established that mineralization does not occur (Eilberg *et al.*, 1975). At the end of the incubation period tissues were removed from the medium and some were quickly rinsed in distilled water, gently blotted, and allowed to air dry. Others were placed in 10% buffered formalin, processed through paraffin embedding, cut without demineralization at 5–6 μm thickness, and stained with 0.1% aqueous toluidine blue or by the Von Kossa silver technique. Air-dried specimens were used to prepare Debye-Scherrer X-ray diffraction patterns, by Dr. A. Hirschman, Department of Anatomy, Downstate Medical Center, Brooklyn, New York.

Results

Chemical analyses

Table I summarizes over-all chemical composition of the invertebrate cartilages, whose variability undoubtedly reflects a) the pleomorphic nature of the respective tissues, especially in respect of their relative cell and matrix content as revealed by histologic analysis (Schaffer, 1930; Person and Philpott, 1969a), and b) differences in physiologic and growth status of the different organisms at the time of sacrifice. The various tissue collagen contents appear to correlate well with the relative amounts of cells and matrix in the respective tissues, as disclosed by histo-

TABLE I

Chemical components of invertebrate cartilage tissues (wet weight basis; average of 3 analyses ± SEM).

	Limulus (gill)	*Eudistylia* (crown & tentacle)	*Loligo* (cranial)	*Busycon* (odontophore)
% RNA	0.60 ± 0.10	1.31 ± 0.09	0.36 ± 0.07	0.09 ± 0.01
% RNA	0.09 ± 0.03	0.16 ± 0.03	0.09 ± 0.01	0.06 ± 0.02
% Collagen	4.32 ± 1.46	19.05 ± 2.17	6.35 ± 0.43	1.01 ± 0.23
% Non-collagenous protein	19.52 ± 3.74	18.73 ± 2.45	10.12 ± 1.94	3.68 ± 1.85
% Lipid	0.11 ± 0.08	0.39 ± 0.17	0.22 ± 0.12	0.80 ± 0.34

logic studies reported by the above authors. Also, while all the tissues contain hydroxyproline, none of the respective collagens have as yet been purified, so that analyses of purified samples are not yet available. The comparisons between tissues and with the vertebrate tendon collagen standard, are therefore not rigorously quantitative and are only suitable for rough comparison purposes. With the above in mind then, *Eudistylia* crown cartilage has most collagen (19%), while *Busycon* odontophore cartilage has least collagen (1%). The collagen contents of the cartilages from *Loligo* (6%) and *Limulus* (4%) are intermediate between the above values. It should also be mentioned that in the case of *Limulus* and *Loligo,* at least, there are varying amounts of chitin (not quantitated) in the cartilages of these animals, as established by our (unpublished) confirmation of earlier observations by Lankester (1884) and Halliburton (1885).

Tables II and III summarize data on lipid composition of the invertebrate cartilage tissues. It can be noted that in contrast with difficulties experienced in lipid extraction of vertebrate cartilages (Irving and Wuthier, 1968), in the present

TABLE II

Lipid components of invertebrate cartilages.

	Limulus (gill)	*Eudistylia* (crown & tentacle)	*Loligo* (cranial)	*Busycon* (odontophore)
1. *Lipids*				
Neutral lipids (% of total)	70.00 ± 12.84	65.31 ± 12.93	64.35 ± 12.35	26.91 ± 12.31
Phospholipids (% of total)	30.00 ± 5.91	34.69 ± 4.43	35.65 ± 4.54	60.00 ± 4.99
				13.09 (unknown associated with phospholipid fraction)
2. *Neutral lipid components*				
% Mono- & diglycerides	3.41 ± 1.14	3.30 ± 1.37	2.38 ± 0.71	1.28 ± 0.36
% Triglycerides	15.89 ± 3.17	10.84 ± 2.81	7.42 ± 1.34	0.71 ± 0.21
% Free fatty acids	9.39 ± 1.49	11.88 ± 9.74	30.13 ± 12.39	6.90 ± 1.45
% Methylated fatty acids	10.09 ± 3.74	6.16 ± 0.94	2.73 ± 1.03	1.95 ± 1.21
% Cholesterol (or steroid-like)	2.89 ± 0.83	11.44 ± 6.31	2.71 ± 0.91	11.96 ± 2.49
% Esters of steroid-like substances	28.56 ± 12.99	21.69 ± 12.49	18.98 ± 2.77	4.11 ± 3.98
3. *Phospholipid components*				
% Phosphatidyl choline	10.00 ± 7.85	3.55 ± 0.99	8.73 ± 1.43	15.11 ± 2.76
% Phosphatidyl serine	1.23 ± 1.01	1.64 ± 0.81	2.00 ± 1.00	13.74 ± 5.84
% Phosphatidyl ethanolamine	0.34 ± 0.12	5.19 ± 2.31	5.40 ± 2.35	10.23 ± 3.97
% Lysolecthicin	0.01 ± 0.01	0.82 ± 0.43	0.78 ± 0.43	1.36 ± 0.49
% Phosphatidic acid	12.32 ± 5.54	20.76 ± 3.94	16.34 ± 7.44	14.73 ± 2.11
% Sphingomyelin	0.89 ± 0.63	1.37 ± 1.10	0.95 ± 0.39	3.71 ± 1.79
4. *Unknown neutral lipids and phospholipids*	4.98	1.37	1.45	14.21

TABLE III

Fatty acid composition of invertebrate cartilage lipids.

Per cent fatty acid	*Limulus* gill cartilage		*Eudistylia* crown cartilage	
	Pooled phospholipids	Pooled neutral lipids	Pooled phospholipids	Pooled neutral lipids
8:0	—	—	—	—
10:0	—	—	0.56	—
12:0	—	—	0.74	2.49
12:1	3.50	2.81	—	9.98
14:0	trace	5.79	3.32	3.99
14:1	5.84	0.92	0.46	4.49
16:0	50.75	37.05	30.19	44.00
16:1	6.08	1.50	4.92	1.99
16:2	trace	trace	0.22	trace
18:0	23.83	28.91	27.47	5.99
18:1	—	trace	13.56	3.99
18:2	—	trace	2.31	—
18:3	trace	—	2.92	—
20:0	—	—	0.68	—
20:1	—	9.36	2.21	1.25
20:2	—	—	1.38	—
20:3	—	—	trace	—
22:0	—	—	0.68	3.49
22:1	—	trace	1.00	trace
22:2	—	—	0.46	—
24:0	—	—	—	—
Unknown	10.00	13.66	6.92	18.35

study, as mentioned in the section on materials and methods, no difficulties were encountered with the lipid extractions of the invertebrate cartilage tissues. This suggests that in the invertebrate tissues, lipids are probably not covalently bound, as in some noncartilaginous vertebrate connective tissues such as those of skin (Rabinowitz and Shapiro, 1973) but exist rather in loose physical associations with other tissue components. Indeed, the lipid patterns shown in Tables II and III resemble patterns of lipid composition reported for noncartilaginous vertebrate connective tissues (Rabinowitz, Luddy, Barford, Herb, Orlean and Cohen, 1967; Rabinowitz, Rutberg, Cohen and Marsh, 1973; Rabinowitz and Hercker, 1974). It is therefore interesting that the invertebrate cartilages do show significant differences in comparison with vertebrate cartilage tissues. Thus, invertebrate tissue levels (Table II) of lecithin, triglycerides and phosphatidyl ethanolamine are much lower, and levels of phosphatidic acid much higher than in vertebrate cartilages (Irving and Wuthier, 1968; Wuthier, 1973). Also noteworthy in this context is the unusually high value for the phosphatidyl serine component of phospholipids found in *Busycon* odontophore cartilage, 13.7%, as compared with values between 1.2–2.0% for the cartilages of *Limulus, Eudistylia* and *Loligo*.

Individual fatty acid determinations were made for the lipids of *Limulus* and *Eudistylia* cartilages (Tables III). The more saturated fatty acids (palmitic, stearic, oleic) were found to be approximately two to three times more abundant than unsaturated fatty acids in these invertebrate cartilages. This is approximately

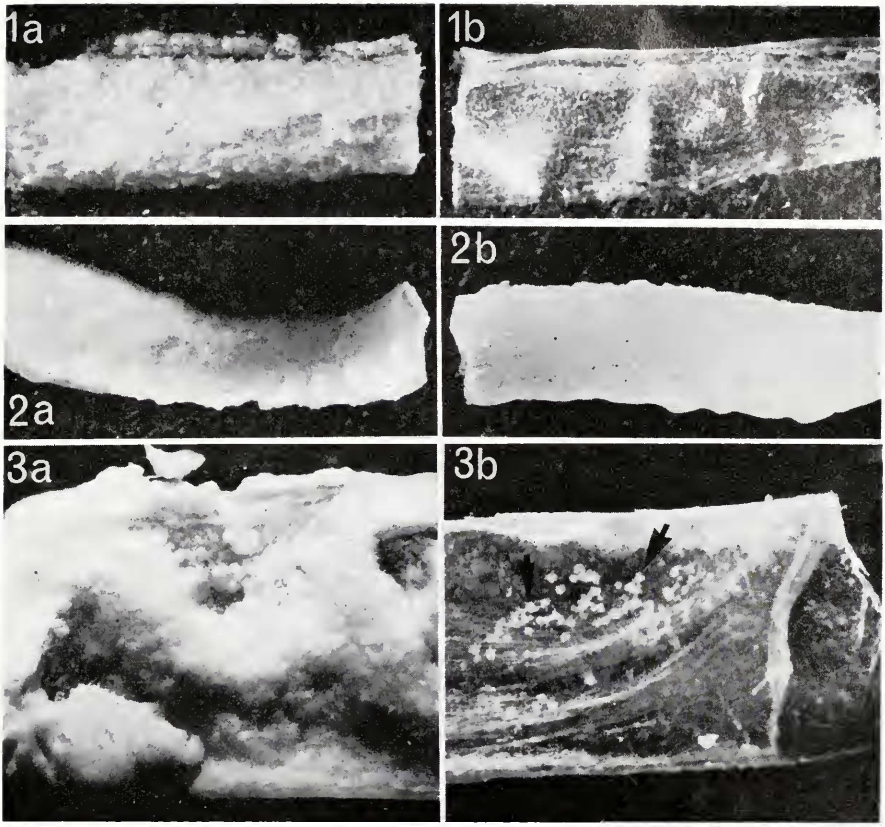
the same ratio observed for lipids of vertebrate collagens (Nikkari and Heikinen, 1968) and is somewhat surprising because in most marine organisms (both vertebrate and invertebrate) unsaturated fatty acids are usually much higher than saturated ones (Markley, 1960). The presence of as yet unidentified lipid components in all tissues is of interest, and attempts will be made to identify these components in future experiments. It may also be noted that significant amounts of methylated fatty acids were found in all tissues. At times, the presence of methylated fatty acids may reflect artifacts of transmethylation from solvent methanol used in extraction procedures. However, Leikola, Nieminen and Salomaa (1965), Louh and Garton (1970), and Rabinowitz, Tavares and Marsh (1972) have shown that nonspurious, significant values for methylated fatty acids are found in a variety of tissues under conditions in which artifacts of transmethylation cannot occur. In the present experiments, the values for methylated fatty acids are believed to be valid because it has been established in earlier experiments that the techniques employed would not lead to transmethylation (Louh and Garton, 1970; Rabinowitz, Tavares and Marsh, 1972).

Mineralization experiments

The *in vitro* mineralization of invertebrate cartilage is visible to the unaided eye as a crystalline phase deposited within and upon the surface of the incubated tissues. The typical appearance of well-mineralized cartilages is illustrated in Figures 1, 2, and 3, which show respectively, air dried specimens of mineralized (a), and non-mineralized (b) controls from *Busycon, Limulus* and *Loligo*, after 96 hours of incubation.

After 10 and 16 hours of incubation, unaided eye examination of tissue specimens from each of the three organisms revealed that mineral deposits were apparently lacking in all. These observations were confirmed by histologic and X-ray diffraction examinations of the 10 and 16 hour-incubated tissues, which also could not detect evidences of mineralization.

After 24 hours of incubation, however, signs of mineralization were detectable by the unaided eye in *Busycon* odontophore cartilage, but not in *Limulus* or *Loligo* cartilages. Staining of alternate serial sections from the 24 hour incubated tissues by toluidine blue and by the Von Kossa technique, confirmed that there was mineralization of the *Busycon* cartilage, but no mineral deposits were seen in sections of either the *Limulus* or *Loligo* cartilages. Figure 4 is a photomicrograph of a toluidine blue-stained section of *Busycon* odontophore cartilage (24 hours incubation) showing the appearance of granular mineral deposits within cells (*mc*), matrix (*mm*) and also within the periochondrium (*mp*). Figures 5 and 6 show, respectively, comparable photomicrographs of toluidine blue-stained sections from *Limulus* and *Loligo* cartilages also after 24 hours incubation. No signs of mineralization are detectable in the latter two specimens. In black and white photomicrographs of toluidine blue stained sections, as shown in Figure 4, mineral appears as black granules. Von Kossa stain of alternate serial sections show positive reactions in these same granules. After 32 hours incubation, first signs of mineralization of both *Limulus* and *Loligo* cartilages were seen. At this time the mineralization of *Busycon* cartilage was much farther advanced than that of *Limulus* or *Loligo*.



FIGURES 1–3. Air-dried specimens of invertebrate cartilages after 96 hours incubation, to illustrate appearance of (*a*) well mineralized and (*b*) non- or poorly-mineralized control tissues. The mineralized and control tissues in each figure were removed from the same animal, and incubated in the same media, except that temperature of incubation was 37° C for mineralized specimens and 20° C for controls. At the latter temperature, mineralization is strongly inhibited or does not occur at all.

FIGURE 1. *a* (*left*) : *Busycon* odontophore cartilage, impregnated and incrusted with white, opaque, hydroxyapatite crystals; *b* (*right*) : translucent appearance of non-mineralized control specimen; seven × magnification.

FIGURE 2. *a* (*left*) : *Limulus* gill cartilage, impregnated and incrusted with white, opaque, hydroxyapatite crystals; *b* (*right*) : non-mineralized control cartilage. The latter tissue surface is smooth, and although the tissue also appears opaque, it is in fact more translucent than the mineralized tissue, but this translucency was not captured by the camera; seven × magnification.

FIGURE 3. *a* (*left*) : *Loligo* cranial cartilage, impregnated and incrusted with white, opaque crystals of hydroxyapatite; *b* (*right*) : control specimen, non-mineralized except for a small number of crystal aggregates (arrows) ; nine × magnification.

After 48 hours incubation, mineralization had progressed in cartilage specimens from all three animals, and was most advanced, still, in *Busycon* tissue, as judged by relative amounts and distribution of mineral. After 96 hours of incubation, mineralization in each of the three tissue types was so widespread, that it was difficult to discern significant differences between them in respect of quantity and dis-

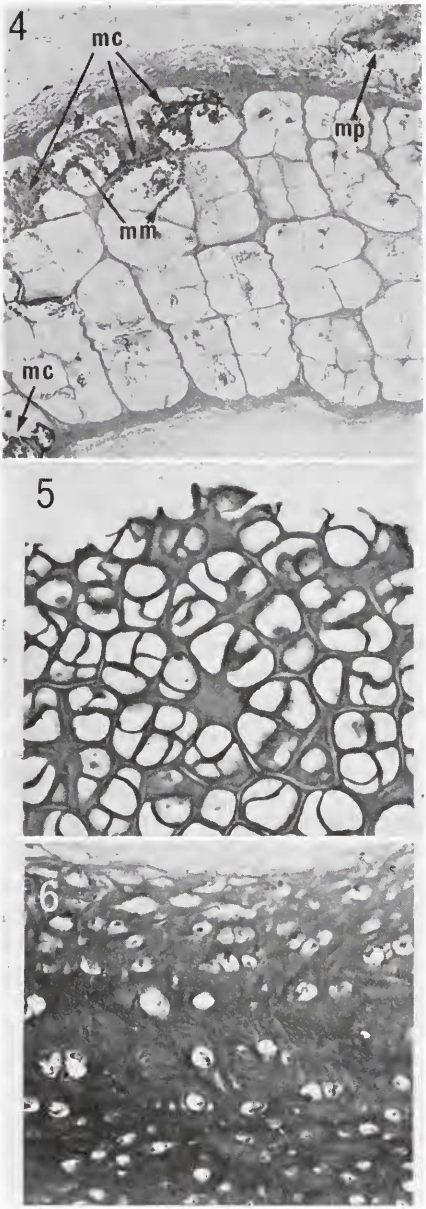


FIGURE 4. Photomicrograph of *Busycon* odontophore cartilage section (24 hours incubation) showing distribution of mineral granules within cells (*mc*), in matrix (*mm*) and in perichondrium (*mp*); toluidine blue stain, 153 × magnification.

FIGURE 5. Photomicrograph of *Limulus* gill cartilage section (24 hours incubation) showing lack of mineral deposition in the tissue; toluidine blue stain, 153 × magnification.

FIGURE 6. Photomicrograph of *Loligo* cranial cartilage section (24 hours incubation) showing lack of mineral deposition in the tissue; toluidine blue stain, 153 × magnification.

tribution of mineral. Detailed studies of chondrocyte and matrix alterations during mineralization, as a function of incubation time, are now in progress and will be reported in a separate paper. X-ray diffractions made from air-dried, well-mineralized (*i.e.*, 96 hour incubation) cartilage specimens from each of the three animals used in this study, gave in each instance a typical pattern of a poorly crystallized biological hydroxyapatite, as illustrated in a preceeding publication (Eilberg, Person and Zuckerberg, 1975, figure B).

Discussion

It is not possible at this time to interpret the variability of lipid and other chemical components reported for the different cartilage tissues, from an evolutionary or phylogenetic standpoint. Only after a larger data base has been accumulated can such an attempt be made. At present, only the differences in collagen content of the respective cartilages appear susceptible of some interpretation, based upon the relative matrix content of the tissues as revealed by histologic studies. Thus, *Eudistylia* cartilage containing most collagen (19%) has large sheets of matrix with relatively few cells (Person and Mathews, 1967). *Loligo* cartilage, next in respect of collagen content (6%) is a typical hyaline cartilage (Philpott and Person, 1970). *Limulus* and *Busycon* cartilages containing 4% and 1% collagen respectively are of the "chondroid" variety of cartilage, and possess decreasing amounts of matrix (Shaffer, 1930; Person and Philpott, 1969b; Person and Philpott, 1967).

Of special interest was the demonstration that while invertebrate cartilages do not mineralize *in vivo* in the course of their natural history, they possess the capability to be induced to mineralize *in vitro*. Two features of this *in vitro* mineralization are worthy of mention here. First, until now only hydroxyapatite mineral phases can be formed in the invertebrate cartilage tissues and not calcium carbonate, which is the mineral of widest natural occurrence and distribution in invertebrate skeletal tissues. However, this may be the result of lack of a suitable metastable medium for calcium carbonate deposition, and the search for such a medium continues. Secondly, our data show that the *in vitro* mineralization by hydroxyapatite occurs earlier and with greater intensity in *Busycon* odontophore cartilage than in either *Limulus* gill or *Loligo* cranial cartilages. This is of interest because *Busycon* cartilage phospholipid content is 13.5% phosphatidyl serine, as compared to 1.2% and 2.0% phosphatidyl serine in *Limulus* and *Loligo* cartilages, respectively. This suggests a possible role for phosphatidyl serine in mineralizability of invertebrate cartilages, perhaps similar to its demonstrated role in mineralization of vertebrate cartilage (Irving and Wuthier, 1968, Wuthier, 1973). Studies designed to answer this question are now in progress.

Finally, it may be suggested that the ability to induce mineralization of invertebrate endoskeletal cartilage tissues creates a new experimental approach to a major problem of animal evolution, namely, the lack of mineralized endoskeletal cartilage structures in invertebrates, as compared with vertebrates. It may now be possible to deal with both genetic and environmental influences which may play roles in repression (or activation, if such can be achieved) of the ability of the invertebrate cartilage tissues to mineralize *in vivo*.

We thank Dr. Richard Libbin and Mr. Rober Ozer for their assistance, and Dr. Albert Hirschman for preparation of X-ray diffraction patterns.

Summary

Cartilage from several invertebrate organisms *Limulus polyphemus* (Arthropoda), *Eudistylia polymorphia* (Annelida), *Busycon canaliculatum* (Mollusca) and *Loligo pealii* (Mollusca) were assayed for RNA, DNA, collagen, noncollagenous protein and lipid content. The variability in levels of collagen (1–19%) in the respective tissues, appeared to correlate well with their relative matrix content as revealed by previous histologic study. Determinations were made of mono- and diglycerides, triglycerides, free fatty acids, methyl esters, steroid-like materials and steroid-like esters, phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, lysolecithin, phosphatidic acid, and sphingomyelin. The fatty acid composition of the major sub-groups was determined, and it was found that the ordinary fatty acids, palmitic, stearic, and oleic represented the largest fraction, whereas values for unsaturated fatty acids in the invertebrate cartilages were smaller than those usually reported for marine organisms. Significant levels of phosphatidyl serine were found in all tissues, being especially high in the odontophore cartilage of *Busycon, i.e.*, 13.7% of phospholipid content. Some unusual and as yet unidentified fatty acids, representing approximately 10% of the total fatty acid composition were also found.

In vitro mineralization of *Busycon* odontophore cartilage, *Limulus* gill cartilage and *Loligo* cranial cartilage were carried out by incubation of the respective tissues at 37° C in a medium metastable for hydroxyapatite. Of the three tissues mentioned above, earliest signs, and most rapid mineralization occurred in the *Busycon* cartilage, which also has the highest content of phosphatidyl serine. This latter observation is of interest because of the demonstrated role of phosphatidyl serine in mineralization of vertebrate cartilages, and suggests the possibility of a similar role in the *in vitro* invertebrate cartilage mineralization.

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